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# Randomly Ordered Addressable High-Density Optical Sensor Arrays

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Array-based sensors provide an architecture for multianalyte sensing. In this paper, we report a new approach for array fabrication. Sensors are made by immobilizing different reactive chemistries on the surfaces of microspheres. Sensor arrays are prepared by randomly distributing a mixture of microsphere sensors on an optical substrate containing thousands of micrometer-scale wells. The sensors occupy a different location from array to array; thus the identity of each sensor is ascertained and registered on the detector using encoding schemes, rather than by a predetermined location in the array. The approach thereby shifts the demand from fabrication to signal processing. The availability of commercial image analysis software makes such a shift both cost-effective and time efficient.

The demand for high-throughput and cost-effective analysis of complex mixtures has driven technology toward the fabrication of compact, high-density array devices. Arrays can be categorized according to their transduction mechanism and include surface acoustic wave sensors,[1−3] microelectrodes,[4−5] solid-state sensors,[6] and fiber-optic sensors,[7−12] all of which have been used for multianalyte measurements. These arrays are fabricated using conventional techniques such as ink-jet printing,[13−15] screen printing, photolithography,[16−22] and photodeposition[7−11] in which the sensing chemistries are applied directly to the sensor surface. Typically, multiple fabrication steps are required which are labor-intensive and subject to some degree of variability. These formats require that the identity of each probe on the array be preregistered by its position.

We report here a new approach to array fabrication that fully exploits both the high information density potential of an optical imaging fiber and rapid signal processing. We have made randomly ordered, addressable, high-density optical sensor arrays by combining optical imaging fibers, selective etching, chemical assays, and optical encoding schemes. The overall concept is shown in Figure 1. An optical imaging fiber is chemically etched to produce a high-density array of micrometer-sized wells. A simple, one-step procedure was developed for dispersing tens of thousands of chemically modified microspheres in an etched fiber to create a high-density sensor array. Using the approach presented in this paper, microsphere arrays are prepared in a random fashion such that each array has a unique sensor pattern (i.e., each sensor in the array occupies a different position from array to array). Optical encoding schemes using fluorescent dyes were developed to identify each sensor in the array.

## EXPERIMENTAL SECTION

**Materials.** Ammonium fluoride and sodium azide were obtained from Aldrich Chemical Co. (Milwaukee, WI); sodium bicarbonate buffer, alkaline phosphatase, glutaraldehyde, phosphate-buffered saline (PBS), bovine serum albumin, *N*-(2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid) (HEPES) buffer, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO); 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid succinimidyl ester, NeutraLite avidin, fluorescein diphosphate (FDP), fluorescein-labeled avidin, fluorescein-labeled biotin, indodicarbocyanine (DiIC), and Texas red cadaverine (TRC) were from Molecular

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EXHIBIT A

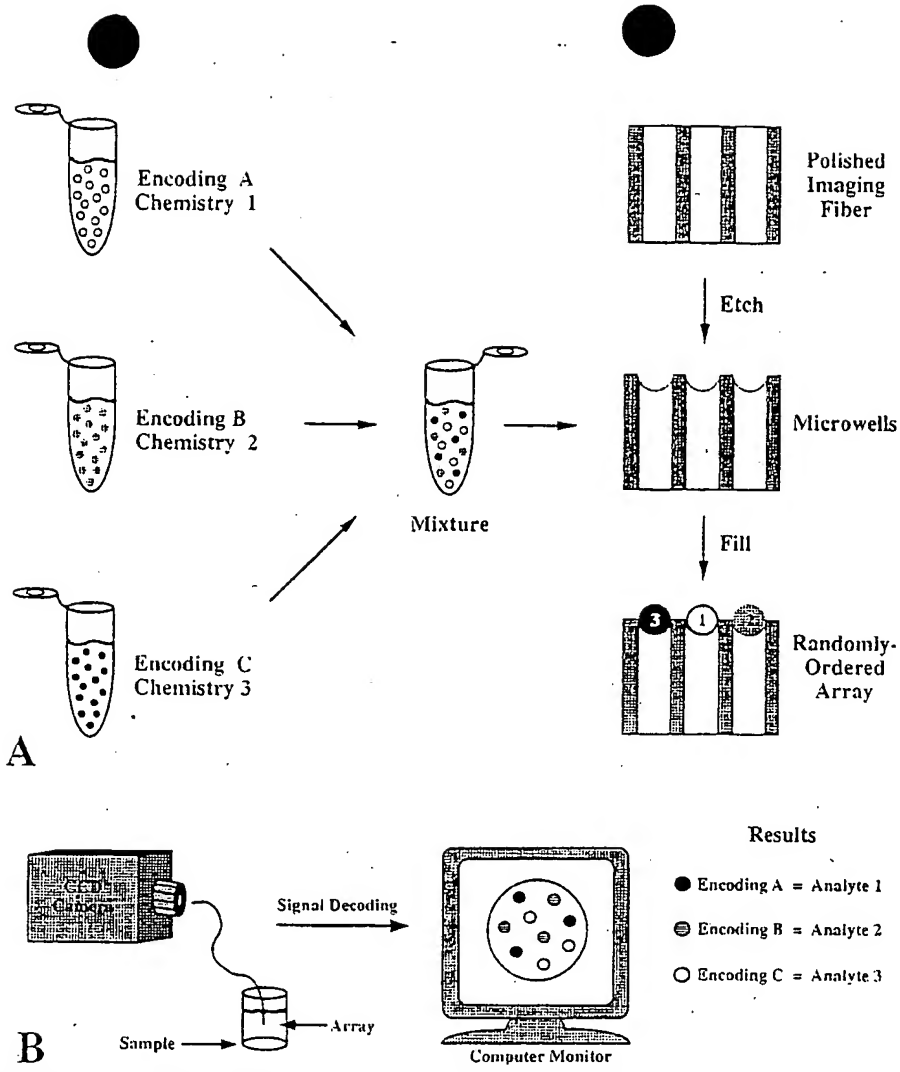


**Figure 1.** Schematic concept of a randomly ordered addressable high-density optical sensor array. (A) Separate sets of individual micrometer-sized sensors are created by covalently immobilizing indicating chemistries on the surface of microspheres. The microspheres are mixed together and randomly distributed in the wells of a chemically etched optical imaging fiber. (B) The array is placed in a sample solution and the chemical nature of each microsphere sensor in the array that results in an analytical signal is ascertained and registered simultaneously on a CCD detector.

Probes (Eugene, OR); hydrofluoric acid was from Fisher Scientific (Fair Lawn, NJ); Tween 20 was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ); poly(methylstyrene)–divinylbenzene microspheres and silica microspheres were from Bangs Laboratories, Inc. (Fishers, IN). All chemicals were used as received.

**Microwell Fabrication.** A 1000-$\mu$m-diameter imaging fiber containing ~20600 individually cladded optical fibers was successively polished using 12-, 9-, 3-, 1- and 0.3-$\mu$m lapping films. The polished fiber was sonicated to remove residual lapping film material and allowed to dry. The distal face of the imaging fiber was submerged in a buffered hydrofluoric acid solution (0.2 g of ammonium fluoride, 100 $\mu$L of hydrofluoric acid (50%), and 600 $\mu$L of deionized water) for 80 s. The fiber was immediately dipped into a beaker of deionized water to stop the reaction. The fiber was then rinsed under running water and sonicated to remove any salts formed in the reaction. **CAUTION: Hydrofluoric acid is extremely corrosive; handle with care.**

**Microsphere Encoding and Addressing.** Three 200-$\mu$L aliquots of stock 3.1-$\mu$m-diameter poly(methylstyrene)–divinylbenzene microspheres ($5.8 \times 10^9$ microspheres/mL) were prerinsed in dimethylformamide. Approximately 200-$\mu$L aliquots of DiIC/TRC solutions made in dimethylformamide [(A) $9.8 \times 10^{-6}$ M/$1.6 \times 10^{-3}$ M; (B) $4.9 \times 10^{-5}$ M/$4 \times 10^{-4}$ M; and (C) $4 \times 10^{-4}$ M/$6 \times 10^{-4}$ M] were added to the microspheres. The microsphere/dye suspensions were agitated for 24 h on a wrist-action shaker. The excess dye solution was removed from each suspension by vacuum filtration using DMF-compatible, 0.5-$\mu$m Fluoropore membrane filters (Millipore Co., Bedford, MA). The filtered microspheres were sonicated and washed by centrifugation in distilled water (containing 0.01% Tween 20) to remove residual dye on the outside of the microspheres.

The individual encoded ratios were distinguished by taking the mean fluorescence intensity minus the background intensity at each emission wavelength and then dividing the two values (670 nm/610 nm) to get the signature of that particular ratio. Signature ranges were determined by performing the above analysis on large sets of individual microspheres. Unique sets of encoding dyes are determined according to the formula $n!/p!\,(n - p)!$, where $n$ is the number of dyes and $p$ is the number of combinations.

**Microsphere Immobilization Chemistries.** All immobilization reactions were performed in aqueous solutions to ensure that

the microspheres would not swell and release or alter the signature of the encoding dyes. Microspheres with surface-bound alkaline phosphatase were prepared using the following protocol. Approximately 2 mg of DiIC:TRC ratio A encoded microspheres were activated with 2.5% glutaraldehyde in 10 mM PBS (138 mM NaCl and 2.7 mM KCl) pH 6.9, containing 0.01% Tween 20 for 2−3 h. The microspheres were then washed twice using deionized water containing 0.01% Tween 20 and twice using 10 mM PBS, pH 7.7, containing 0.01% Tween 20. The microspheres were then allowed to react with ~150 μL of alkaline phosphatase solution (1 mg/mL in 10 mM PBS, pH 7.7, containing 0.01% Tween 20) in an ice bath. After 3 h, the microspheres were washed three times using 10 mM PBS, pH 7.4, containing 0.01% Tween 20. The microspheres were then treated with a filtered solution of 0.5% bovine serum albumin for ~3 h to passivate the exposed microsphere surface. Finally, the microspheres were washed three times in PBS, pH 7.4, containing 0.01% Tween 20.

The following procedure was used to biotinylate the microsphere surfaces. An 18 μM stock solution of 6-((6-((biotinoyl)-amino)hexanoyl)amino)hexanoic acid, succinimidyl ester, was prepared in dimethyl sulfoxide. The stock NHS-biotin was then diluted 1:10 in 0.15 M sodium bicarbonate buffer, pH 8.3, containing 0.01% Tween 20. Approximately 2 mg of microspheres encoded with DiIC:TRC ratio B were treated with ~300 μL of the dilute NHS-biotin solution. After 30 min, the microspheres were washed three times in 10 mM PBS, pH 7.4, containing 0.01% Tween 20. The microspheres were then treated with a filtered solution of 0.5% bovine serum albumin for ~3 h to passivate the exposed microsphere surface. Finally, the microspheres were washed three times in PBS, pH 7.4, containing 0.01% Tween 20.

Avidin-labeled microspheres were fabricated using the following method. First, ratio C encoded microspheres were biotinylated as described above. A 5 mg/mL stock solution of NeutraLite avidin was prepared in 10 mM PBS, pH 7.4, containing 2 mM sodium azide. Approximately 2 mg of ratio C encoded microspheres were treated with 60 μL of stock NeutraLite avidin in 240 μL of 10 mM PBS, pH 7.4, containing 0.01% Tween 20 at 4 °C. After 24 h, the microspheres were washed three times using 10 mM PBS, pH 7.4, containing 0.01% Tween 20.

**Fluorescence Measurements.** Typical FDP solutions were 0.045 mM in 10 mM HEPES buffer, pH 8.6, containing 50% glycerol. Typical fluorescein-labeled avidin working solutions used were 1−10 μg/mL concentrations in 0.15 M sodium bicarbonate buffer, pH 8.3, containing 0.01% Tween 20. Typical fluorescein-labeled biotin working solutions were ~12 μM in 0.15 M sodium bicarbonate buffer, pH 8.3, containing 0.01% Tween 20.

**Imaging Instrumentation.** Atomic force microscopy was performed in the contact mode using a Digital Instruments Dimension 3000 scanning probe microscope (Santa Barbara, CA). All fluorescence measurements are made using a modified epifluorescence microscope coupled to a CCD-detector.[23] White light from a 75-W xenon arc lamp is collimated, passed through an excitation filter, and reflected by a dichroic mirror. The reflected light is collected by a 10× or 20× microscope objective and focused onto the fiber's proximal face. The excitation light then propagates through each individual optical channel in the fiber to the distal face where the encoded and modified microspheres fluoresce in the presence of a target molecule. The fluorescence returning to the fiber's proximal face is collected by the microscope objective, transmitted through the dichroic mirror, passed through an emission filter, and detected by a CCD camera. Data processing is performed using IP Lab from Signal Analytics Co. (Vienna, VA).

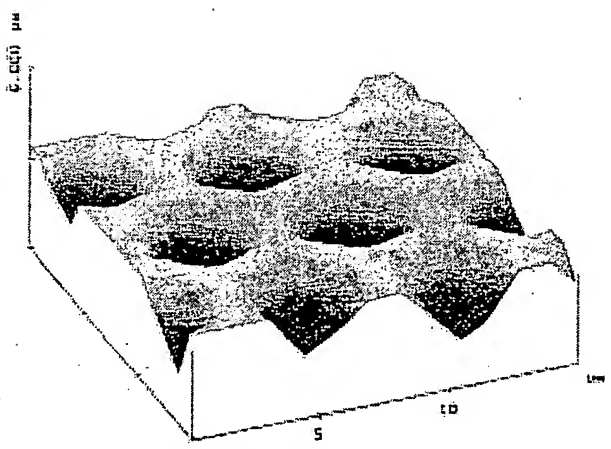

**Figure 2.** Atomic force micrograph of ~3.6-μm-diameter microwells fabricated by chemically etching a polished, 1000-μm-diameter imaging fiber. The microwells are ~3 μm deep and have a volume of ~36 fL.

## RESULTS AND DISCUSSION

**Microwell Fabrication.** Optical imaging fibers are commercially available and comprise thousands (e.g., 3000−100 000) of hexagonally packed, individual optical fibers. During fabrication, the individual fibers are melted and drawn together in a coherent manner such that each fiber in the array carries its own, isolated optical signal from one end of the fiber to the other.[24-25] Previously, we have fabricated micro- and nanowell arrays using optical imaging fibers as an architectural template.[26] A wet chemical etching procedure is used to selectively etch the cores of the individual optical fibers by taking advantage of the difference in etch rates between the core and cladding materials.[27] By carefully controlling the etching time, high-density, ordered microwell arrays of known shape and volume are obtained. Since the well architecture is determined by the preformed imaging fiber, a high packing density of microwells is easily and reproducibly fabricated in tens of seconds without requiring a high degree of precision. In the present work, we use a 1000-μm-diameter imaging fiber containing ~20600 individual, 3.6-μm-diameter optical fibers. Figure 2 is an atomic force micrograph (AFM) of microwells fabricated by submerging the distal face of the imaging fiber into a buffered hydrofluoric acid solution for 80 s. The microwells produced are ~3 μm deep and have a volume of ~36 fL ($3.6 \times 10^{-14}$ L). Since every microwell is connected to its own optical fiber, each well can be interrogated as an individual sensor.


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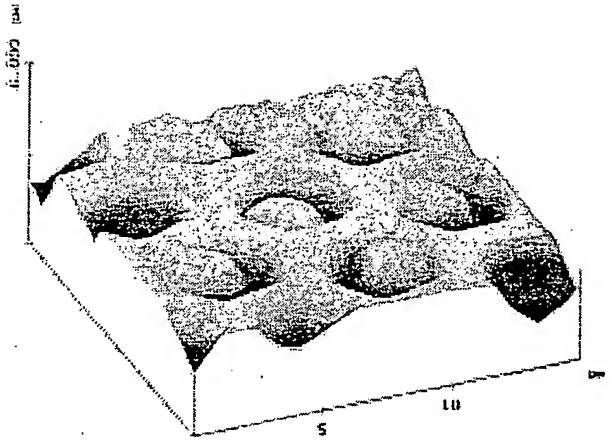

**Figure 3.** Atomic force micrograph of ~3.6-$\mu$m-diameter microwells containing a single 3.1-$\mu$m-diameter microsphere in each microwell.

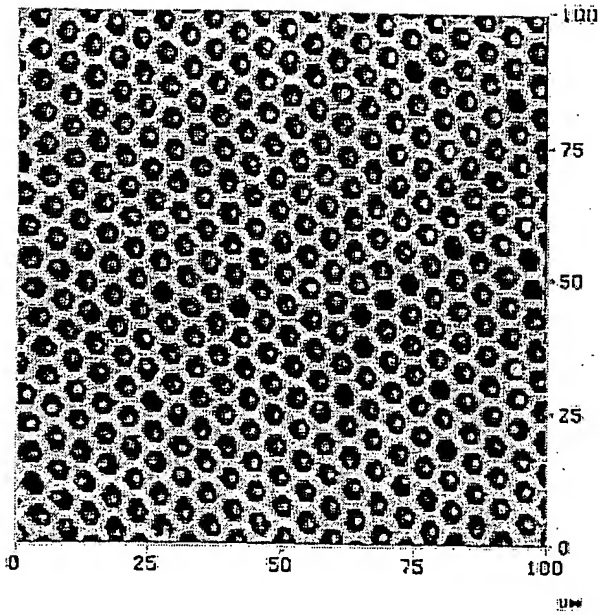


**Figure 4.** Atomic force micrograph of a high-density microwell array containing 1 microsphere/microwell with 96% microsphere packing density.

**Array Fabrication.** We have developed a simple, one-step procedure for dispersing tens of thousands of individual microspheres in an etched fiber to create a high-density sensor array (Figure 1). A dilute suspension ($\leq 3.9 \times 10^6$ microspheres/$\mu$L) is prepared containing monodisperse microspheres with diameters and volumes slightly smaller than those of the etched microwells. The microwell array fiber is held vertically in a fiber chuck and a 1-$\mu$L drop of the suspension is pipetted onto the fiber's distal face. Since the microsphere and well diameters are complementary, individual microspheres randomly settle into each well (Figure 3) as the solution evaporates (≤5 min at room temperature). Excess microspheres are removed using a foam swab. Once the microspheres are dispersed in the microwells, they remain in place. This may be due to an electrostatic attraction between the untreated glass surface of the wells and the amino-functionalized microspheres. Alternatively, a thin polymer film can be used to fix the microspheres in place, although experiments have shown this to be unnecessary. Using this simple procedure, ~96% of the microwells in the array can be filled with microspheres (Figure 4), affording a high-density array of nearly 20 000 individual sensors on a single 1000-$\mu$m-diameter optical imaging fiber. If more sensors are required, the arrays can be easily bundled. This technique is highly flexible since both the microspheres and the optical imaging fibers are available in a wide variety of sizes and can thus accommodate many different applications.

**Microsphere Encoding and Addressing.** Conventionally, high-density arrays are prepared in a directed, patterned fabrication process that provides positional registration. Using the approach presented in this paper, microsphere arrays are prepared in a random fashion such that each array has a unique sensor pattern (i.e., each sensor in the array occupies a different position from array to array). To differentiate one sensor in the array from another, we employ encoding schemes using fluorescent reporter dyes that are spectrally resolved. Each sensor is encoded with a particular dye signature that enables it to be registered on a detector and identified. For example, a two-dye system provides the ability to encode microspheres using different concentration ratios of the two dyes, as shown schematically in Figure 1. A similar method has been used previously in flow cytometry.[28] Nonoptical encoding schemes have been employed for analysis of combinatorial syntheses on solid supports.[29-31] Microspheres are encoded simply by soaking them in organic solvents containing multiple dyes in different ratios. The solvent swells the polymeric microspheres and allows the dyes to penetrate into the microspheres' cores. Excess solvent is removed by vacuum filtration, entrapping the dyes in the hydrophobic interior regions of the microspheres. This procedure is performed easily with minimal sophistication and provides a ratiometric measurement that is insensitive to light intensity or detector sensitivity changes. DiIC and TRC were chosen as the two-dye system because both dyes can be excited at one wavelength (577 nm) and give rise to separate emission wavelengths (670 and 610 nm, respectively). These dyes are also advantageous because their wavelengths are distinct from fluorescein (ex 490 nm/em 530 nm), which is used as our target reporter dye for the initial proof of concept. Poly(methylstyrene)−divinylbenzene microspheres were separately encoded with three DiIC/TRC concentration ratios (A−C) producing sets of microspheres with fluorescence intensities in a distinct or "signature" range as shown in Table 1. The concentration ratios of each dye set listed in Table 1 are the dye concentrations in the soaking solution. Using commercially available microscopy analysis software, these different spectral signatures can be defined and counted automatically in several seconds with the optical detection system. The number of resolvable signatures for a given dye pair is determined by the broadness of the ratio range. Utilizing the present two-dye

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**Table 1. Encoding Scheme for Sensor Identification**

| encoding dye ratio (DiIC/TRC) | dye concn ratio in solution (DiIC/TRC) | microsphere signature range (670 nm/610 nm) |
|---|---|---|
| A | $9.8 \times 10^{-6}$ M/$1.6 \times 10^{-3}$ M | 0.19–0.25 |
| B | $4.9 \times 10^{-5}$ M/$4.0 \times 10^{-4}$ M | 0.32–0.56 |
| C | $4.0 \times 10^{-4}$ M/$6.0 \times 10^{-4}$ M | 0.82–2.3 |

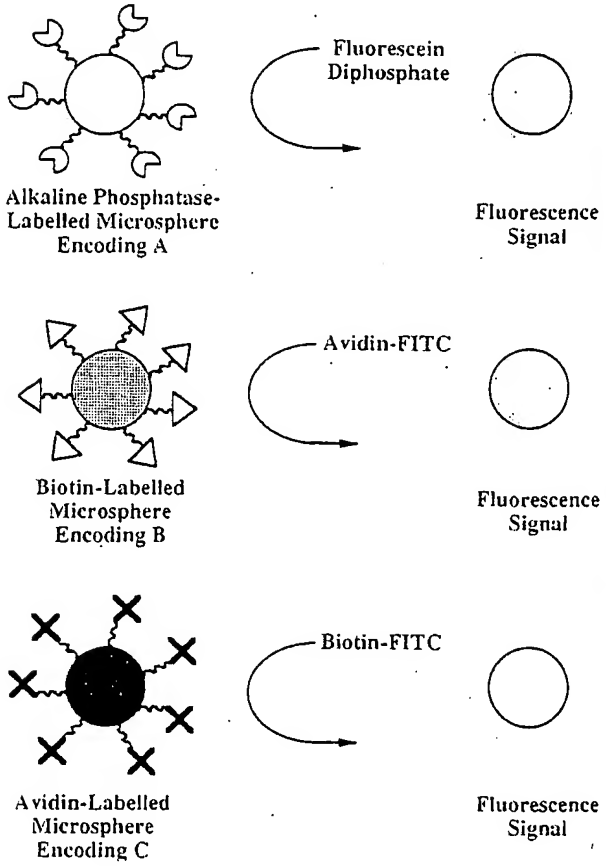


**Figure 5.** Schematic representation of the sensing chemistries immobilized on each different set of encoded microspheres and their response to target molecules.

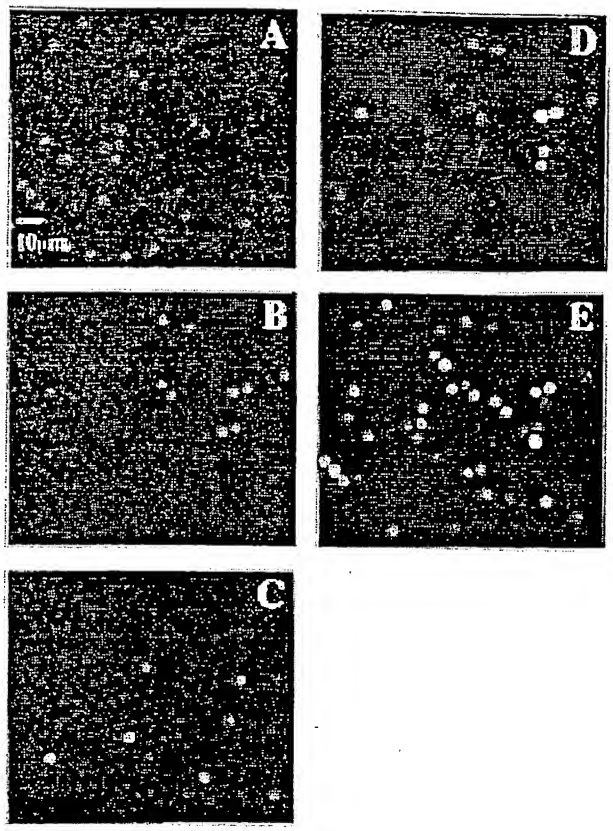


**Figure 6.** (A) Fluorescence response of alkaline phosphatase-bound microspheres to fluorescein diphosphate. (B) Fluorescence response of avidin-bound microspheres to fluorescein-labeled biotin. (C) Fluorescence response of biotinylated microspheres to fluorescein-labeled avidin. Image A was acquired with a 2-s CCD acquisition time at 530 nm using 490-nm excitation light. Images B and C were acquired with a 1-s CCD acquisition time at 530 nm using 490-nm excitation light. (D) Fluorescence image of encoded microspheres taken at 670 nm using 577-nm excitation light. (E) Fluorescence image of encoded microspheres taken at 610 nm using 577-nm excitation light. Both images D and E were acquired with a 5-s CCD acquisition time. All images were background-subtracted, normalized, and contrast adjusted for image clarity. In all images, white indicates high fluorescence intensities.

encoding scheme, we can conservatively prepare five distinct ratio pairs; however, with improvements in the encoding procedure and the exploration of new dye pairs, resolution of at least 10 ratio pairs is expected. Greater diversity can be achieved by employing other encoding dyes in binary, ternary, or higher combinations and by using more discriminants (e.g., intensity and microsphere size). As an example, employing 6 different dyes would provide 15 binary combinations. If 10 ratio pairs can be achieved with each combination, then 150 distinct optical signatures could be identified.

**Microsphere Immobilization Chemistries.** After the microspheres have been encoded, various sensing chemistries are immobilized on the outer surfaces of the microspheres.[28,32-34] Since microspheres are available with various polymer compositions and surface functionalities, a large and diverse microsphere sensor library is envisioned. Furthermore, all the chemistry is performed on large numbers of microspheres ($5.8 \times 10^9$ microspheres/mL) simultaneously, resulting in low sensor-to-sensor variability.

We demonstrated the concept by fabricating a randomly ordered, addressable, high-density optical sensor array for the detection of three independent analytes. First, amino-functionalized, monodisperse ($3.1 \pm 0.045\ \mu$m) poly(methylstyrene) divinylbenzene microspheres were encoded with DiIC/TRC ratios A–C as described above. Each set of microspheres was modified with a specific sensing chemistry using amine functionalities on the microsphere surface (Figure 5). Microspheres encoded with dye ratio A were activated with glutaraldehyde and coupled to alkaline phosphatase. In the presence of the substrate FDP, these

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microspheres produce a localized, enzyme-generated fluorescence signal. Microspheres encoded with dye ratio B were biotinylated using NHS-biotin. These microspheres give a fluorescence signal in the presence of fluorescein-labeled avidin. Finally, microspheres encoded with dye ratio C were treated with avidin. These microspheres give a fluorescence signal in the presence of fluorescein-labeled biotin. A sensor array was prepared by mixing quantities of each encoded and modified microsphere set and randomly dispersing them in the microwells. To visually demonstrate the technique, a sparsely populated array was fabricated for fluorescence measurements.

**Fluorescence Measurements.** Measurements are made using a modified epifluorescence microscope coupled to a CCD detector,[23] allowing all the individual optical fibers to be viewed and interrogated simultaneously without scanning. To clearly demonstrate that the three types of modified microspheres elicit separate responses, the array was exposed to FDP, fluorescein-labeled avidin, and fluorescein-labeled biotin sequentially. The reactions were monitored with 1- or 2-s acquisition times at 530 nm using 490-nm excitation light, followed by a buffer wash to remove any unreacted target molecules. Binding or enzyme activity occurred in <10 s, giving rise to localized fluorescence signals (Figure 6A–C). The microspheres yielding an analytical signal were decoded by switching to a second excitation wavelength (577 nm) to excite the encoding dyes. The emission ratio of 670 nm/610 nm (Figure 6D and E) was used to determine the identity of each microsphere. In the designated region of interest, 39 microspheres were decoded. Thirty-six fell within the correct signature range, yielding a 92% accuracy. In this series of experiments, the array was exposed to six different solutions (three target molecules and three buffer washes); neither loss nor rearrangement of the microspheres was observed with the application of multiple liquid samples. Note, however, that a key feature of this technology is that the identity of each microsphere is not determined by its position in the array but by its encoded signature (i.e., any unforeseen microsphere relocation would be immaterial). Occasionally, a single microwell entraps two microspheres. When decoded, the resulting well gives a signal outside the range of any single microsphere. The probability of a false positive or negative result is further diminished because there are multiple copies of each microsphere in a single array.

**Future Directions: Randomly Ordered Nanowell Arrays.** The trend in sensor technology is toward fabrication of ever smaller array devices to minimize sample volumes and increase sensitivity. Previously, we showed that the packing density of an array can be increased by tapering the imaging fibers before etching.[26] For example, the packing density of the microwell array used in the above experiments is $2 \times 10^7$ wells/cm$^2$ (Figure 7A). By tapering the same imaging fiber, a nanowell array with a packing density of $> 4.4 \times 10^9$ wells/cm$^2$ is obtained (Figure 7B). The individual well diameters are decreased from ~3.6 $\mu$m to ~200 nm. To demonstrate the flexibility of the present technique, we have created a "nanosphere" array by placing ~300-nm-diameter silica spheres in an etched, tapered imaging fiber containing ~500-nm-diameter wells (Figure 7C). These results clearly show the versatility of this new approach to array fabrication and the potential for designing very small devices.

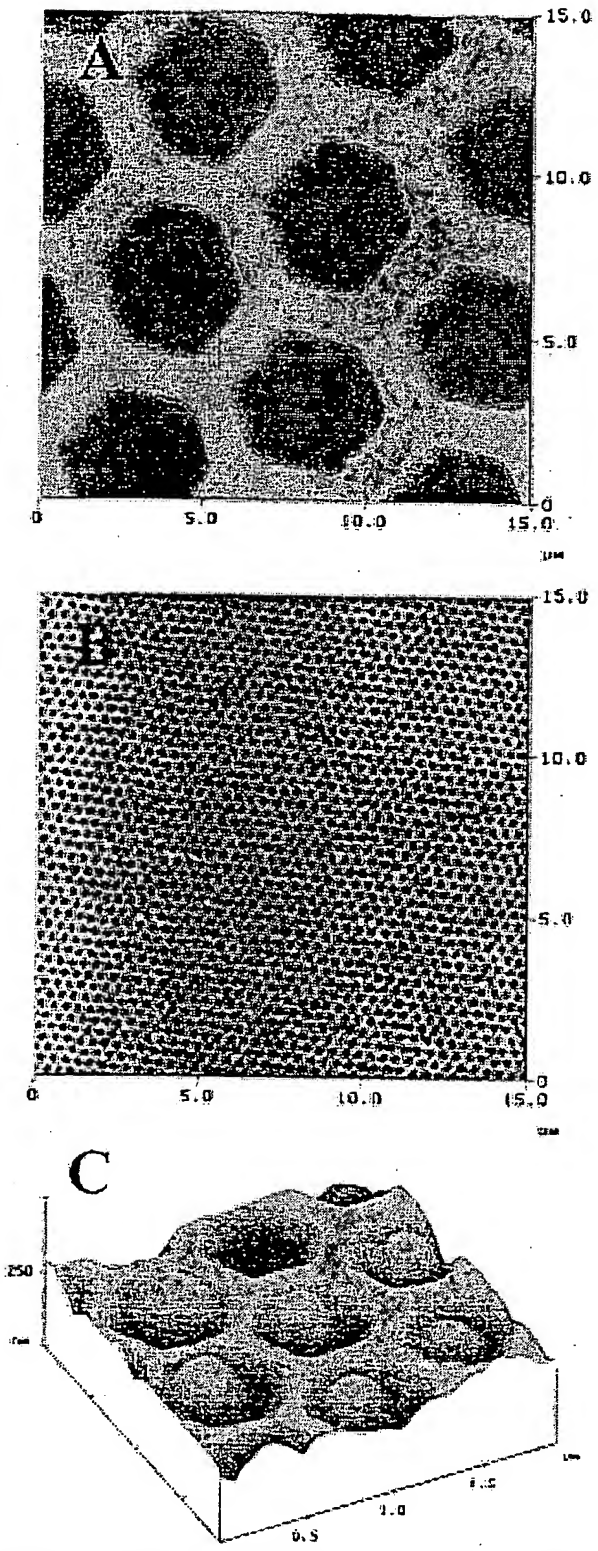


**Figure 7.** Atomic force micrograph of an etched imaging fiber before (A) and after (B) tapering. The individual well diameters were decreased from ~3.6 $\mu$m (A) to ~200 nm (B). (C) Atomic force micrograph of ~500-nm wells containing ~300 nm silica spheres.

## CONCLUSION

We have demonstrated the ability to fabricate randomly ordered, addressable, high-density optical sensor arrays. This

approach to preparing array sensors offers a dramatic shift from conventional sensor fabrication procedures which require multiple steps such as photolithography, micromachining, and site-selective syntheses. Microwell arrays are easily and reproducibly fabricated using commercially available imaging fibers without requiring a high degree of precision. One series of chemical reactions can create a stock supply of billions ($5.8 \times 10^9$ microspheres/mL) of chemically modified sensors with virtually identical properties that can be used in the fabrication of new arrays for different analyte sets. Also, the need to identify every location and to calibrate each sensor in the array is eliminated because only those microspheres giving rise to an analytical signal need to be decoded. This advantage may be of particular value when rapid diagnostic tools are required or when analyses with low "hits" are performed, such as immunodiagnostics or low-frequency mutation analysis with gene arrays. Multiple copies of each sensor are easily represented in each array, providing a level of redundancy that should avoid both false positives and false negatives. The image processing software used to analyze the spectral information makes this approach advantageous in applications requiring high sample throughput. Since the fabrication process is fast and simple and the materials are inexpensive, there is no economical demand to reuse the array. We are presently extending this work to demonstrate arrays for immunoassays, gene probe sequences, and vapor sensors. Finally, the sensors offer the ultimate flexibility—as new assays come along, new microspheres simply can be added to the existing microsphere mixture at virtually no setup or time cost.


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